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Nerium oleander indirect leaf photosynthesis and light harvesting reductions after clipping injury or *Spodoptera eridania* herbivory: High sensitivity to injury

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ABSTRACT

Variable indirect photosynthetic rate (P_n) responses occur on injured leaves after insect herbivory. It is important to understand factors that influence indirect P_n reductions after injury. The current study examines the relationship between gas exchange and chlorophyll a fluorescence parameters with injury intensity (% single leaf tissue removal) from clipping or *Spodoptera eridania* Stoll (Noctuidae) herbivory on *Nerium oleander* L. (Apocynaceae). Two experiments showed intercellular [CO_2] increases but P_n and stomatal conductance reductions with increasing injury intensity, suggesting non-stomatal P_n limitation. Also, P_n recovery was incomplete at 3 d post-injury. This is the first report of a negative exponential P_n impairment function with leaf injury intensity to suggest high *N. oleander* leaf sensitivity to indirect P_n impairment. Negative linear functions occurred between most other gas exchange and chlorophyll a fluorescence parameters with injury intensity. The degree of light harvesting impairment increased with injury intensity via lower (1) photochemical efficiency indicated lower energy transfer efficiency from reaction centers to PSII, (2) photochemical quenching indicated reaction center closure, and (3) electron transport rates indicated less energy traveling through PSII. Future studies can examine additional mechanisms (mesophyll conductance, carbon fixation, and cardenolide induction) to cause *N. oleander* indirect leaf P_n reductions after injury.

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1. Introduction

Plants allocate resources to growth, maintenance, and reproduction, while also using resources to resist or tolerate abiotic and biotic stresses. When a constitutive plant defense (e.g., secondary metabolites) is breached, resources have already been allocated to the failed constitutive defense and additional resources may be allocated to induced defense responses. In cases where resource allocation to chemical defenses limits resource allocation to compensatory responses to injury, this may help explain a trade-off between plant chemical defense and growth [1] or tolerance [2] that can have fitness consequences [3,4]. Indirect P_n reduction (reduced activity on remaining tissue near sites of herbivory injury) has been suggested to result from a secondary metabolism trade-off with primary physiology, since resources allocated to chemical defense are unavailable for, or cause downregulation of, photosynthesis [5–7; but see 8]. Yet, only a subset of plant species tested have indirect P_n reductions from defoliation herbivory [9–12], and variable responses can occur within a single species [8]. The degree of chemical defense investment by a plant can correlate with degree of indirect P_n reductions after herbivory [6; but see 8], so chemically well defended plants may be more prone to experience indirect P_n reductions after herbivory. Studying individual leaf P_n responses to injury are relevant because leaves are important for mediating whole plant responses to herbivory [13] and P_n is a highly sensitive leaf response assay to herbivory [10,14]. At the scale of a leaf one can study mechanisms by which injury affects photosynthesis on the leaf and neighboring uninjured (or regrowth) leaf P_n responses [9].

Plant responses to herbivory can be studied by comparing injured leaf responses across an injury intensity continuum relative to responses of uninjured leaves (zero injury intensity). Plant damage response curves indicate whether a plant performance parameter changes after injury and the relevant range(s) of injury intensity if the parameter changes. Several theoretical damage response functions (Fig. 1) are possible [derived from 15]: overcompensation ('1'), tolerance ('2'), tolerance at low injury intensities that transitions to negative linear reductions at higher injury levels

Abbreviations: ETR, electron transport rate; C_i, intercellular [CO_2]; JA, jasmonic acid; F'_V/F'_M, light-adapted leaf maximal photochemical efficiency; F'_M, maximal light-adapted leaf fluorescence; F'_O, minimal light-adapted leaf fluorescence; q_P, photochemical quenching; P_n, net photosynthetic rate; PSI, photosystem I; PSII, photosystem II; PI, post-injury; SAW, *Spodoptera eridania*-southern armyworm; F'_S, steady state light-adapted leaf fluorescence; g_s, stomatal conductance; F'_V, variable light-adapted leaf fluorescence.

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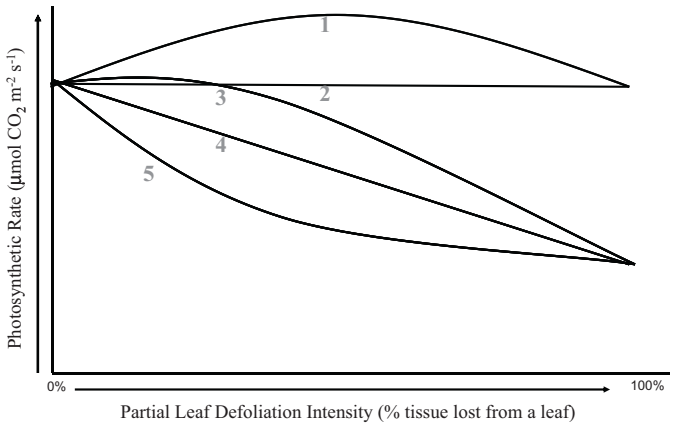

Fig. 1. Some basic theoretical leaf photosynthetic response curves to single leaf injury intensity are shown to represent different major leaf responses to injury. These functions include: (1) overcompensatory function (positive slope over part of the injury range), (2) photosynthetic tolerance function (slope of zero), (3) compensatory function (initial slope of zero transitioning to a negative slope), (4) negative linear function (consistent negative slope), and (5) high injury sensitivity function (initial highly negative slope transitioning to a slope near zero).

('3'), consistent parameter decreases with each unit of injury ('4'), and high injury sensitivity at low injury levels ('5') transitioning to little additional reduction at higher injury levels. The meaning of a damage response curve depends on the scale of the parameters. Damage response curves were initially studied to examine yield relationships with defoliation intensity at a field scale [16], which indicated the range of injury at which a particular crop was sensitive to yield loss and helped to develop economic injury levels [15,17]. Damage response curves applied to the scale of individual plants [17] are used to study plant tolerance and fitness consequences of injury [18]. Here the functions are considered for a single leaf (Fig. 1), where P_n is the dependent variable examined in response to % single leaf tissue loss as the independent variable to indicate the degree of P_n change that occurs on remaining tissue of an injured leaf [12,14,19–21]. This informs us about leaf P_n sensitivity to change after injury and at which range(s) of tissue loss P_n has large (or small) changes per unit of injury. Several leaf gas exchange and chlorophyll a fluorescence parameters can also be measured to provide insights about why P_n changes [22], and which parameters are most closely associated with P_n changes after injury [6,23].

1.1. Study species

Nerium oleander L. (Apocynaceae) is an evergreen perennial dicot shrub/bush native to Mediterranean regions, but has been planted in most tropical and subtropical regions globally. It is grown as a yard and street median ornamental plant in southern USA states [24]. Common oleander shrubs vary from 1 to 6 m in height and have variable numbers (~5–100) of branches. Many complete, narrow lanceolate leaves occur along a branch. A single leaf (see Fig. 2A) can be 5–21 cm long, 1–4 cm at the widest part of the leaf, and cover 10–40 cm^2 (personal observation). Leaf drop is rare, so a leaf can remain on a plant >1 year. Only a few specialist insect and no vertebrate herbivores feed on *N. oleander*. This is because *N. oleander* is a chemically well defended plant that contains high total cardenolide levels and specific compounds like oleandrin and neriine [25]. Oligophagous specialist defoliators of *N. oleander* inside the USA include larvae of the oleander polka dot moth (*Syntomeida epilais* Walker) [24] and the spotted oleander moth (*Empyreuma affinis* Rothschild) [26], while outside the USA there are the oleander hawkmoth (*Daphnis nerii* L.) and common crow butterfly (*Euploea core* Cramer). These four herbivore species were not observed at the study site. However, the generalist southern armyworm moth (SAW; *Spodoptera eridania* Stoll) has been observed to feed on *N. oleander* (personal observation) and was able to be used in one experiment.

1.2. Research and hypothesis

I chose to study *N. oleander* leaf physiological responses to herbivory because it is a chemically well-defended species. Delaney [21] showed that mechanical clipping injury to single *N. oleander* leaves resulted in indirect P_n reductions on single injured leaves that had 50% tissue removal. Yet, what are the mechanisms that contribute to injured leaf indirect P_n reduction? The reported experiments extend Delaney [21] by examining *N. oleander* gas exchange and chlorophyll a fluorescence parameter response curves across a single leaf injury intensity continuum (% tissue loss from a single leaf), using clipping injury in two experiments and SAW larval herbivory in a third. Since P_n reductions had already been documented on injured *N. oleander* leaves [21], neither P_n overcompensatory ('1' from Fig. 1) nor tolerance ('2' from Fig. 1) response curves were expected. The specific objectives addressed with the reported experiments were: (1) to determine which leaf P_n theoretical responses ('3', '4', or '5' from Fig. 1) apply to *N. oleander* after clipping injury or SAW herbivory and (2) to compare the P_n relationship to leaf injury intensity with other gas exchange and light-adapted leaf chlorophyll fluorescence parameters. Mechanical injury is useful to examine leaf time course response to injury because the injury is imposed immediately, so post-injury (PI) gas exchange measurements in the first 120 min are relative to a specific injury time. Insect herbivory is spread out over temporal scales of minutes or hours, so it is extremely difficult in the first 120 min post-injury to be compared with a specific injury reference time. Gas exchange parameters were measured to indicate whether injury leads to stomatal or non-stomatal limitations to photosynthesis, and chlorophyll a fluorescence parameters provided insights about how injury affected light harvesting reactions. Non-stomatal limitations to photosynthesis after herbivory can include mesophyll limitations due to light harvesting impairment [6,8,12,23,27–30], and impairment of photosynthetic carboxylation reactions [20,30,31].

2. Materials and methods

Experimental *N. oleander* plants were located ~500 m north of the Xavier University of Louisiana (XULA) campus in New Orleans, LA. Plants received ambient precipitation, which is 150 cm annually for New Orleans. I was able to limit photosynthetic measurements to one leaf from a given branch on an oleander plant, and each plant was used in only one experiment.

2.1. Clipping injury

Baseline and post-injury photosynthesis measurements were collected with an infra-red gas analyzer. The measurement location (1.6 cm diameter circle) was located halfway along the length of each *N. oleander* leaf whether it remained uninjured (Fig. 2A) or subsequently had tissue removal (Fig. 2B–D). Baseline measurements were collected and immediately followed by clipping injury on the measured leaf in each of two experiments. A ruler was used to measure leaf length and width; leaves included in the experiment were 12–15 cm long and 3–4 cm wide. Leaf clipping with scissors removed ~10% of the length on one side of a leaf without any midrib injury to result in a ~5% photosynthetic tissue removal section (Fig. 2B). When there was >1 tissue removal section, a ~2 mm wide strip of tissue separated each tissue removal section (Fig. 2C and D). Leaves with ≤45% tissue removal had all removal sections located along the same side of the leaf relative

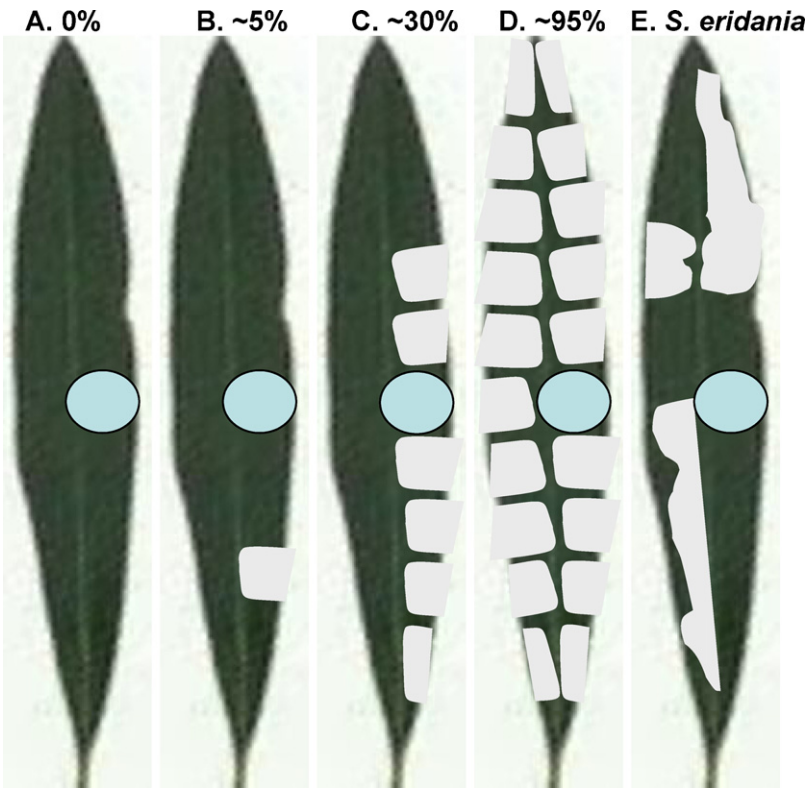

Fig. 2. Examples of a *N. oleander* leaf with different tissue loss intensities superimposed using white, and a circle showing where photosynthetic measurements were collection from a common location at the middle of the leaf. Examples of leaf injury intensities include: (A) no tissue removed (uninjured), (B) approximately 5% tissue removal by clipping injury, (C) approximately 30% tissue removal by clipping injury, (D) approximately 95% tissue removal by clipping injury, and (E) representation of tissue removal by SAW feeding.

to the midrib and one central space was kept for photosynthesis measurements (e.g., Fig. 2C). Leaves with ≥50% tissue removal had the first 9 sections located on the side of leaf where photosynthesis measurements were collected and additional sections were located on the other side of the leaf's midrib for a maximum of ~95% tissue removal (e.g., Fig. 2D). While gas exchange and chlorophyll a fluorescence were measured from one leaf, clipping injury was imposed on the previously measured leaf. Thus, pre-injury baseline measurements were measured within a few minutes before clipping injury was imposed (important to allow for 1.5 h post-injury (PI) measurements in the 2005 clipping experiment below). Many apparently healthy looking leaves had $P_n \sim 1$–$3\ \mu mol\ m^{-2}\ s^{-1}$ (personal observation). Thus, leaves were screened during pre-injury measurements to verify that they were photosynthetically active ($P_n \geq 14$, 10, and $7.5\ \mu mol\ m^{-2}\ s^{-1}$ in the 2004 and 2005 clipping, and 2005 SAW experiments, respectively). The minimal acceptable level was lowest for the 2005 SAW experiment because ambient air temperature ~37–38 °C, and many leaves had lower photosynthetic activity or had shut down.

2.2. Clipping experimental details

The 2004 clipping experiment involved pre-injury *N. oleander* leaf photosynthesis measurements from 1300 to 1500 h on July 16, 2004, 1 d post injury measurements from 1300 to 1500 h on July 17, and 3 d post-injury measurements from 1300 to 1500 h on July 19. A negative linear function ('3' from Fig. 1) was most commonly reported for indirect P_n reduction due to increasing stomatal limitation with % single leaf tissue loss [12,19,20], so that was expected here for P_n and other photosynthetic parameters. If photosynthetic recovery occurs by 3 d post-injury, then a function should have a significantly less negative (or non-significant) slope at 3 d PI than at 1 d PI. A light intensity of $1000\ \mu mol\ m^{-2}\ s^{-1}$ reached leaves based on a model LI-190 quantum sensor (LiCor Biosciences Inc.) on the measurement chamber, so this was set as the within measurement chamber light intensity for all three measurement times. Leaf clipping injury intensity ranged from 0% to 90% in 10% increments, where three leaves received each injury intensity so 30 leaves were measured in this experiment. Each of three plants received one of each of the 10 injury intensities for a total of 10 treatment leaves on each plant, each treatment leaf occurred on a separate branch on its plant, and treatment leaves were located on the distal half of a branch. All branches occurred on the same side of the plant facing sunlight during photosynthesis measurements and the three experimental plants were adjacent in a row of trees.

The 2004 clipping experiment involved 1 d and 3 d PI measurements, commonly measured post-injury intervals. Yet, leaf P_n can rapidly change in response to injury [12,21]. Thus, the 2005 clipping experiment involved pre-injury *N. oleander* leaf photosynthesis measurements from 1400 to 1530 h and added 1.5 h PI measurements from 1530 to 1700 h on May 2, 2005; 1 d PI measurements from 1500 to 1530 h on May 3; and 3 d PI measurements from 1400 to 1530 h on May 5. Light intensities of 1500–$2000\ \mu mol\ m^{-2}\ s^{-1}$ (full sunlight) reached leaves (LI-190 quantum sensor), so $1500\ \mu mol\ m^{-2}\ s^{-1}$ was set as the within measurement chamber light intensity for all four measurement times. After a leaf baseline measurement was collected, a single leaf each received tissue loss levels of 5%, 15%, 25%, 35%, 45%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, and 95%, two leaves received 10%, 20%, 30%, 40%, and 50% tissue loss levels, and four uninjured (0% tissue loss) leaves were measured. These 28 leaves were randomized along branches of two plants, one leaf was measured from each branch, and there were 14 branches with a measured leaf on each plant.

2.3. SAW herbivory experimental details

After an egg mass was collected from *N. oleander* and brought into the lab, larvae hatched and were fed exclusively *N. oleander* leaves placed into plastic water tubes to slow down drying. A mix of 4th and 5th instar SAW larvae were used in this experiment. Several larvae were kept after this experiment, reared to adult emergence, and identified as SAW (P. Martinat, personal communication). SAW larvae can be found rarely feeding on *N. oleander* outdoors (personal observation). Insect herbivory can have more severe effects on leaf P_n than clipping injury [8,12], so negative linear functions after SAW herbivory were expected with steeper slopes than occurred from clipping injury in the first two experiments. The SAW experiment involved pre-injury *N. oleander* leaf photosynthesis measurements from 1130 to 1300 h on June 24, 2005, and 1 d PI measurements from 1130 to 1300 h on June 25. Measurements at 1.5 h PI were not collected because feeding was not completed, and 3 d PI measurements were not collected because leaves were collected immediately after 1 d PI measurements for cardenolide induction analysis. After each baseline photosynthetic measurement was collected from a *N. oleander* leaf, a mesh fabric cage was used to entirely surround the leaf with one 4th or 5th instar SAW larva. Each mesh cage was taped along the opening near the leaf petiole to keep each caterpillar on its designated leaf for 24 h. The use of the two instars allowed for greater variation in the amount of tissue consumption across herbivory treatment leaves, tissue removal occurred anywhere along the leaf (e.g., Fig. 2E), and % tissue removal from SAW larval feeding was visually estimated on each leaf. Uninjured control leaves lacked an SAW larva but were surrounded by a mesh cage. In most cases, the location where baseline photosynthetic measurements were collected was the same location to be measured after leaf herbivory (e.g., Fig. 2E). Full sunlight reached leaves, so a light intensity of 1500 μmol m^{-2} s^{-1} was set within the measurement chamber for both measurement times. One branch on a *N. oleander* plant contained a SAW leaf and a second branch contained a control leaf, and nine plants were used in experiment 3.

2.4. Gas exchange and chlorophyll a fluorescence

Measurements were collected using an open system infrared gas analyzer (model LI-6400; LiCor Biosciences Inc., Lincoln, Nebraska, USA) using a simultaneous gas exchange and chlorophyll a fluorescence chamber (model LI-6400-40) that measures 2 cm^2 (Fig. 2). A 90% red:10% blue wavelength light ratio was provided onto leaf tissue within the chamber, and a CO_2 mixer maintained reference line [CO_2] at 400 μmol CO_2 (mol air)$^{-1}$. No chamber temperature control was used, and air flow was set to 250 μmol m^{-2} s^{-1}. The following gas exchange parameters were measured and reported: P_n (μmol CO_2 m^{-2} s^{-1}), g_s (mol H_2O m^{-2} s^{-1}), and C_i (μmol CO_2 (mol air)$^{-1}$). The following settings were used to collect chlorophyll a fluorescence measurements [32]. A dark pulse involved blue/red actinic light and far red light on together for 1 s. The dark pulse turned off blue and red light while far red light was kept on for an additional 4 s to preferentially excite PSI to allow PSII oxidization. Finally, the far red light was turned off for 1 s to allow for F'_O estimation with modulation set at 250 Hz and a measurement filter of 1 Hz. A saturating flash of 0.8 s followed the dark pulse, where flash intensity was set at 7 (out of 10) to generate >6700 μmol photons m^{-2} s^{-1} centered around 630 nm red light. Flash modulation was set at 20 kHz with a flash filter of 50 Hz. These settings were used during the flash to saturate photochemical activity to estimate F'_M and F_S. Calculated light-adapted leaf fluorescence parameters were $F'_V/F'_M((F'_M - F'_O)/F'_M)$, $q_P(F'_M - F_S)/(F'_M - F'_O)$, and ETR ($\Phi$PSII $\times$ 0.5 (proportion of absorbed quanta by PSII in C3 plants) $\times$ I (saturating light intensity) $\times$ 0.85 (leaf absorbance)) [32].

2.5. Data analysis

All data analyses were performed using SAS version 9.2 [33]. Each of three experiments was analyzed using repeated measures GLM (general linear model) for each gas exchange and chlorophyll a fluorescence parameter. Since each leaf was measured twice in the 2005 SAW experiment, repeated measures GLM still applies to compare pre- and post-injury measurements. The % single leaf clipping tissue removal (leaf injury intensity) was analyzed as a continuous main effect, with a linear term to examine whether a significant function slope existed. No parameters had non-linear functions in the 2004 clipping experiment so a quadratic term was not included in analyses, but some parameters had non-linear functions in the 2005 clipping and SAW experiments so quadratic terms were included in all analyses. Least squares linear regression was subsequently performed with data for most photosynthetic parameters in both clipping experiments and the herbivory experiment (baseline, 1.5 h, 1 d, and 3 d PI depending on the experiment). For parameters with non-linear functions in the 2005 clipping and SAW experiments, SigmaPlot 11.0 was used to determine the best fit function to data.

No significant relationships of any photosynthetic parameter with leaf injury intensity were expected ('2' from Fig. 1) for baseline measures since these were measured immediately before injury occurred, but note that this would not indicate photosynthetic tolerance. Pre-injury levels of photosynthetic parameters were measured to provide for comparison with post-injury values, to verify whether all leaves had the same starting level, and to account for starting levels that differed among leaves. Also, values of uninjured leaves can change over time in response to abiotic conditions, so this needs to be taken into account when inferring photosynthetic changes due to leaf injury. If no significant relationship of a photosynthetic parameter occurred with injury intensity at 1.5 h or 1 d PI this would indicate photosynthetic tolerance ('2' in Fig. 2), while at 3 d PI it would indicate photosynthetic recovery (if change happened at 1.5 h or 1 d PI). If injury significantly affected a photosynthetic parameter at a PI measurement time(s), then that parameter should have a significant date $\times$ linear injury intensity term to indicate a negative slope significantly different from zero and the slope of the corresponding pre-injury baseline function. This applies even to the 2005 SAW experiment, to indicate whether a post-injury function was significantly different from a pre-injury function for each parameter. If a parameter only had a significant date $\times$ linear injury term, then '4' would result (Fig. 2). If a parameter also had a significant date $\times$ quadratic linear injury intensity term that would indicate a non-linear response where the function's slope became significantly more ('3') or less ('5') negative (Fig. 2), and that this rate of slope change was greater for a post-injury than pre-injury function. Individual linear least squares regression (including quadratic injury term for some parameters) was subsequently performed to examine specific relationships of each parameter with injury intensity at each measurement time.

With the SAW experiment, the date $\times$ injury intensity term was not always significant (e.g., for C_i) despite a significant linear regression function at 1 d PI. To examine how SAW herbivory qualitatively affected *N. oleander* leaf photosynthetic parameters, repeated measures ANOVA was conducted with the presence or absence of SAW on a leaf as a fixed factor, and date was a fixed repeated measures factor. A significant date $\times$ injury term would indicate that SAW herbivory significantly increased or decreased a photosynthetic parameter to a greater extent after injury compared to uninjured control leaves. Fisher's protected LSD post hoc tests were used to

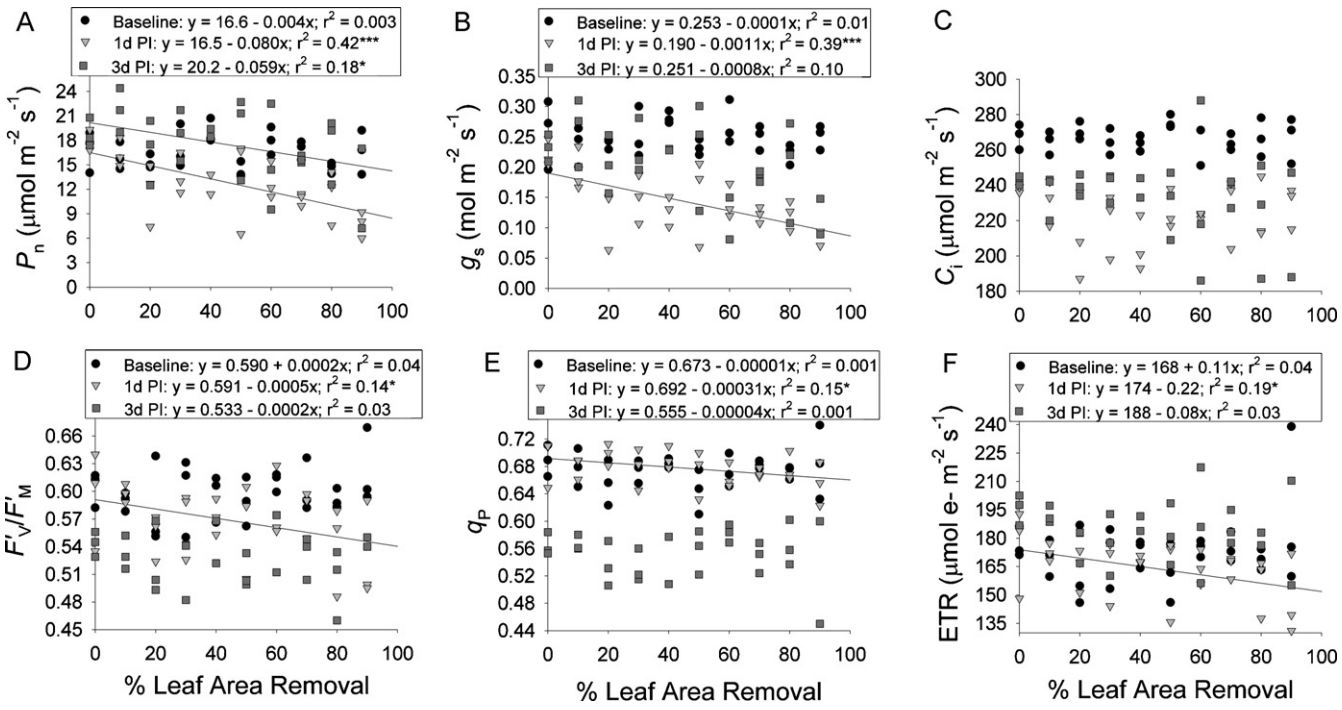

Fig. 3. The relationships for experiment #1 *N. oleander* leaf photosynthesis parameters ((A) P_n, (B) g_s, (C) C_i, (D) F'_V/F'_M, (E) q_P, and (F) ETR) with clipping leaf injury intensity are shown for baseline values immediately before injury (Base, ●), 1 d PI (1dPI, ▽), and 3 d PI (3dPI, ■). The equations of the best-fit functions are presented when at least one slope at one time was significantly different from zero, but only significant functions are shown in graphs.

determine differences between pre- and post injury periods on control and SAW-injured leaves. For all analyses $\alpha = 0.05$.

3. Results

3.1. Clipping injury

3.1.1. 2004 experiment

All parameters lacked a significant relationship between pre-injury baseline measures with leaf injury intensity as expected (Fig. 3A–F). A significant date × injury intensity term occurred for P_n, g_s, F'_V/F'_M, and ETR (Table 1), reflecting significantly different slopes between pre-injury and post-injury functions. Specifically, 1 d PI functions had a significantly more negative slope when compared to non-significant slopes from a corresponding pre-injury function (Fig. 3A, B, D and F). Only P_n still had a significantly negative linear function at 3 d PI (Fig. 3A). Both C_i and q_P had non-significant date × injury intensity and linear injury intensity terms (Table 1). For C_i this was because the 1 d and 3 d PI function slopes were also not significant (Fig. 3C). In contrast, even though q_P had a significantly negative function at 1 d PI (Fig. 3E), the slope of that function was not significantly more negative than the slope from the pre-injury function. The functions of parameters with leaf injury intensity accounted for 14–42% of data variation (r^2) at 1 d PI, but only 0.1–18% at 3 d PI (Fig. 3). All parameters had a significant date term to indicate that measures differed across the measurement times (Table 1), reflecting that other conditions besides leaf injury influenced these photosynthetic parameters.

3.1.2. 2005 experiment

All photosynthetic parameters lacked a significant relationship between pre-injury measures with leaf injury intensity (Fig. 4A–F). For P_n and g_s, both date × linear and date × quadratic leaf injury intensity terms were significant (Table 2). These GLM results occurred because negative exponential ('5' from Fig. 1) functions at 1.5 h and 1 d PI had significantly more negative initial slopes and curvature (significant rate of slope change) than corresponding pre-injury functions (Fig. 4A and B). By 3 d PI, P_n but not g_s had a significantly negative linear function with leaf injury intensity (Fig. 4A and B). Only the date × linear leaf injury intensity term was significant for C_i and ETR (Table 2). For C_i the 1.5 h PI function had a significantly more positive slope than its pre-injury function, but

Table 1
Results from GLM repeated measures analyses of several leaf gas exchange and chlorophyll fluorescence parameters from the 2004 clipping experiment. The *F*-statistic is shown from repeated measures GLM with clipping leaf injury intensity (injury) as a continuous factor, date as a repeated measures fixed factor (date), and the date × injury interaction term.

A. GLM terms	P_n	g_s	C_i	F'_V/F'_M	q_P	ETR
$Injury_{1,28df}$	13^{***}	7.7^{**}	ns	ns	ns	ns
$Date_{2,54df}$	5.7^{**}	5.9^{**}	19^{***}	16^{***}	39^{***}	6.05^{**}
$Date \times injury_{2,54df}$	5.9^{**}	3.4^{*}	ns	5.2^{**}	ns	4.2^{*}

ns: $P > 0.05$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 2
Results from GLM repeated measures analyses of several leaf gas exchange and chlorophyll fluorescence parameters from the 2005 clipping experiment. The *F*-statistic is shown from repeated measures GLM with clipping leaf injury intensity linear (InjLin) and quadratic (InjQuad) as continuous factors, date as a repeated measures fixed factor (date), and date × InjLin and date × InjQuad interaction terms.

A. GLM terms	P_n	g_s	C_i	F'_V/F'_M	q_P	ETR
$InjLin_{1,28df}$	16^{***}	18^{***}	$3.7^{0.065}$	ns	ns	ns
$InjQuad_{1,28df}$	8.0^{**}	14^{***}	7.1^{**}	ns	ns	ns
$Date_{3,74df}$	7.1^{***}	8.1^{***}	ns	ns	ns	ns
$Date \times InjLin_{3,74df}$	9.5^{***}	5.7^{**}	2.9^{*}	ns	ns	3.1^{*}
$Date \times InjQuad_{3,74df}$	5.1^{**}	2.9^{*}	ns	ns	ns	ns

ns: $P > 0.05$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

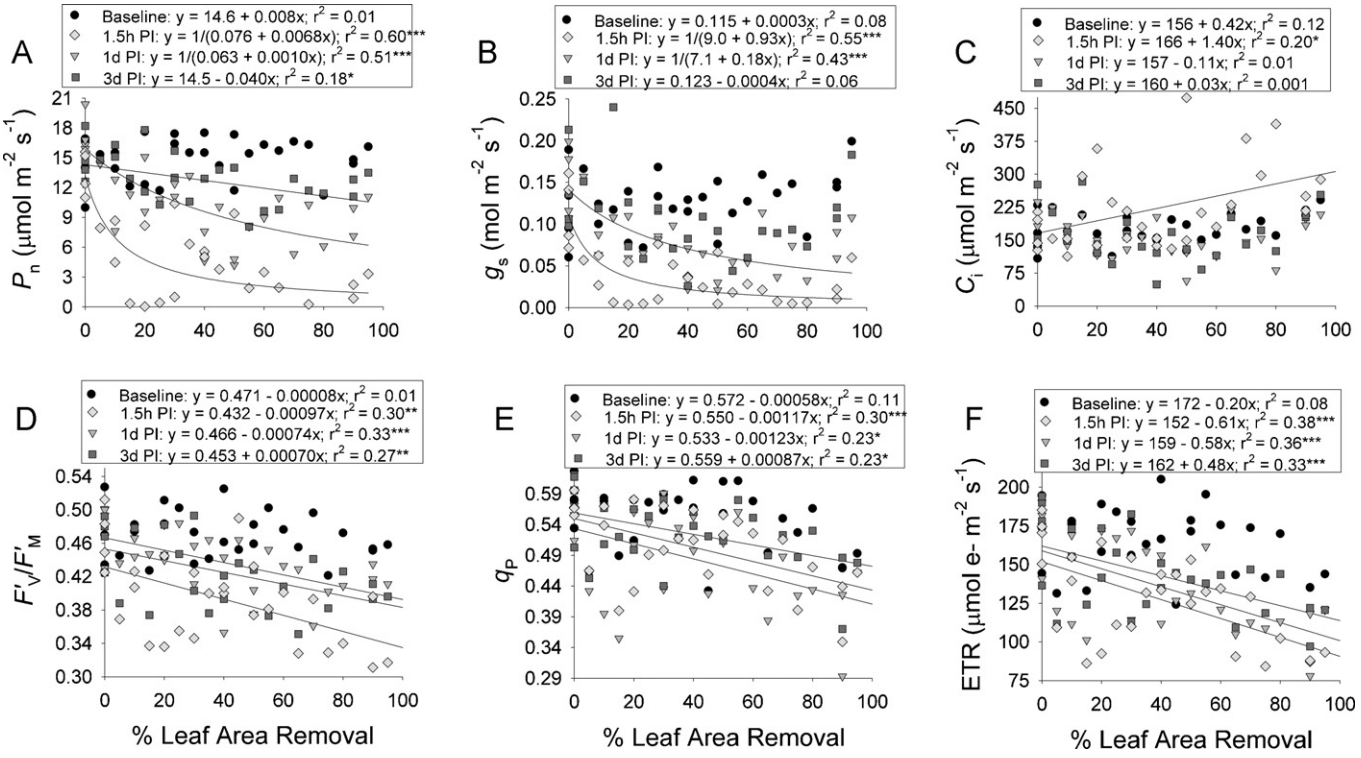

Fig. 4. The relationships for experiment #2 *N. oleander* leaf photosynthesis parameters ((A) P_n, (B) g_s, (C) C_i, (D) F'_V/F'_M, (E) q_P, and (F) ETR) with clipping leaf injury intensity are shown for baseline values immediately before injury (Base, ●), 1.5 h PI (1hPI, ◊), 1 d PI (1dPI, ▽), and 3 d PI (3dPI, ■). The equations of the best-fit functions are presented when at least one slope at one time was significantly different from zero, but only significant functions are shown in graphs.

not functions at 1 d or 3 d PI (Fig. 4C). With ETR, functions at 1.5 h, 1 d, and 3 d PI each had a significantly more negative slope than the pre-injury function (Fig. 4F). Finally, for F'_V/F'_M and q_P there were no significant date × linear leaf injury intensity or linear leaf injury intensity terms (Table 2). Even though F'_V/F'_M and q_P had significantly negative linear functions at 1.5 h, 1 d, and 3 d PI with injury intensity (Fig. 4D and E), those slopes were not significantly more negative when compared to slopes from pre-injury functions. Both P_n and g_s had significant date terms (Table 2) to indicate clearly different levels across measurement dates (Fig. 4A and B), but not for other parameters (Fig. 4C–F).

3.2. SAW herbivory

No parameter had a significant pre-injury function with linear injury intensity (Fig. 5). Both P_n and g_s had significant date × linear and date × quadratic leaf injury intensity terms (Table 3), reflecting significantly different slopes and curvature between pre- and post-injury functions. Specifically, P_n and g_s negative exponential functions at 1 d PI had significantly more negative initial slopes and curvature than pre-injury functions (Fig. 5A and B). For P_n, 7/9 uninjured control leaves had higher levels at 1 d PI than all leaves with SAW herbivory, so the negative exponential function indicated a large decrease in P_n from any level of injury (Fig. 5A). However, only 3/9 uninjured control leaves had higher g_s than all injured leaves (Fig. 5B), so some uninjured leaves maintained higher P_n despite drops in g_s. The date × linear leaf injury intensity term was not significant but there was a significant linear leaf injury term for C_i (Table 3). This was because the 1 d PI C_i function had a significantly positive slope that was not significantly greater than the slope of the pre-injury function (Fig. 5C). The chlorophyll a fluorescence parameters (F'_V/F'_M, q_P, and ETR) all had a significant date × linear leaf injury intensity term (Table 3). For q_P, and ETR, this was because 1 d PI linear functions were significantly more negative than corresponding pre-injury functions (Fig. 3E and F). In contrast, for F'_V/F'_M neither pre- nor post-injury slopes were significant but the 1 d PI function slope was significantly more negative than the pre-injury function slope (Fig. 3D). For all parameters including C_i, when pre-injury and 1 d PI means were compared between uninjured and injured leaves, leaves with SAW herbivory had significantly greater decreases for most parameters (Table 3), or increases for C_i, than uninjured leaves (Table 3). Thus, there was a qualitative impact of SAW herbivory that impaired all photosynthetic parameters (Table 3).

4. Discussion

4.1. Gas exchange and leaf sensitivity to injury

Negative exponential functions with leaf injury intensity best described the data in the 2005 clipping and SAW experiments for P_n and g_s ('5' from Fig. 1), but there was a negative linear function in the 2004 clipping experiment ('4' from Fig. 1). Variation in indirect P_n sensitivity (negative exponential vs. negative linear) to small tissue loss might be influenced by environmental factors a plant faces, type of injury (clipping vs. insect feeding), possibly competing leaf defense and compensatory responses, and the mechanisms driving such responses. I observed variation in leaf photosynthetic sensitivity to impairment from injury for *N. oleander* primarily based on the degree of indirect P_n and g_s impairment of remaining tissue when a leaf received low injury levels. Herbivory by SAW resulted in steeper photosynthetic functions in one experiment compared to clipping injury in two experiments, as herbivory more severely impacts leaf photosynthesis than mechanical clipping [12]. Shallow or even steeply negative exponential functions between indirect leaf P_n reductions (outside infected area) and infection area have been reported for leaf pathogens [e.g., 34–36]. Yet, leaf defoliation does not even always lead to indirect

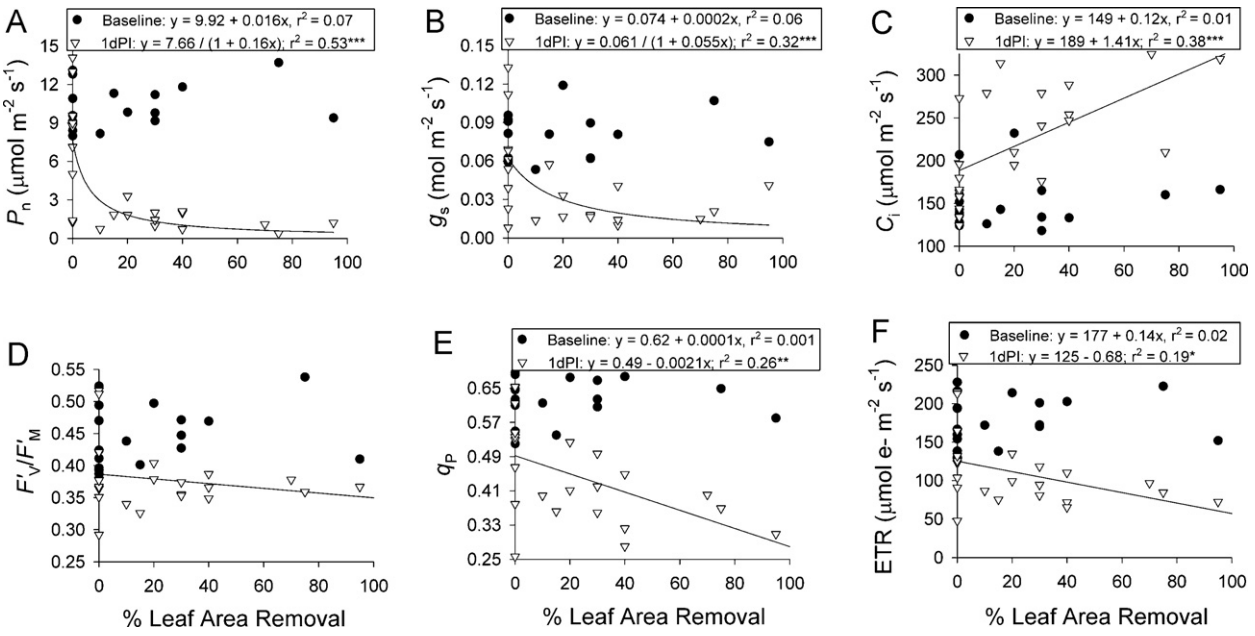

Fig. 5. The relationships for experiment #3 *N. oleander* leaf photosynthesis parameters ((A) P_n, (B) g_s, (C) C_i, (D) F'_V/F'_M, (E) q_P, and (F) ETR) with % leaf tissue area removal from *S. eridania* herbivory for baseline values before injury (Base, ●) and 1 d PI (1dPI, ▽). The equations of the best-fit functions are presented when at least one slope at one time was significantly different from zero, but only significant functions are shown in graphs.

P_n reductions [8–12,22,28,37,38], so for several plant species P_n tolerance to defoliation may be common ('2' and early portion of '3' in Fig. 1). Reported indirect photosynthetic reduction functions have usually involved a negative linear relationship ('4' in Fig. 1) [12,19–21], though negative sigmoid [14] and overcompensatory [19] functions have also been reported. The *N. oleander* results in this study are the first reported negative exponential functions ('5' in Fig. 1) between P_n and single leaf tissue removal intensity, even after clipping injury. Thus, indirect P_n reductions have been detected on injured [21, current study] and nearby uninjured [21] *N. oleander* leaves, suggesting that this plant has little capacity to withstand injury at the scale of an individual leaf.

A negative linear relationship between P_n and injury intensity shows that each unit of tissue removal results in the same degree of indirect P_n reduction on remaining leaf tissue, while a negative exponential relationship suggests that a small amount of tissue loss results in a signal strong enough to cause large indirect P_n impairment on the rest of the injured leaf. Injury contributes to a signal to cause P_n impairment, whether the signal be a mechanical outcome from injury (e.g., cell wall fragments or toxic chemical release), an induced chemical hormone(s) (JA and/or ethylene), or an electrical-hydraulic pulse [39,40]. JA induction has been hypothesized to cause chemical defense induction and photosynthetic gene downregulation to explain a trade-off between photosynthesis and defense responses to injury [7,41]. The peak induction of JA has been reported to occur ~1 h after the application of insect regurgitant to a leaf [42] and within 15 min after burning a nearby leaf [39]. In contrast, the peak emission for ethylene gas is 2 h [42]. Decreases in P_n have been detected 1 h [11] and 1.5 h PI [21; 2005 clipping experiment] after clipping injury, so inhibition via JA induction would seem likely. However, JA has been suggested to downregulate photosynthetic gene transcription to cause indirect P_n impairment [7], a process taking several hours. Stomatal closure occurred within 5 min and P_n reduction 7 min after burning of a nearby leaf, responses that are faster than that reported for JA induction [39]. JA induction and photosynthesis related gene transcript downregulation may influence leaf P_n, but likely only at several h post-injury. Thus, JA downregulation of photosynthesis genes may be too slow to serve as the initial cause of indirect P_n reductions within 1.5 h PI.

Table 3
Results from GLM repeated measures analyses of several leaf gas exchange and chlorophyll fluorescence parameters from the 2005 SAW experiment. (A) The *F*-statistic is shown from repeated measures GLM with SAW leaf injury intensity linear (InjLin) and quadratic (InjQuad) as continuous factors, date as a repeated measures fixed factor (date), and date × InjLin and date × InjQuad interaction terms. (B) The means (±1 SE) for photosynthetic parameters are shown for uninjured (Con) and SAW herbivory (Inj) leaves at pre-injury (Base) and 1 d PI (1dPI) times. Treatments with the same letter are not statistically distinguishable by Fisher's protected LSD post hoc tests.

Parameter	P_n	g_s	C_i	F'_V/F'_M	q_P	ETR
(A) $InjLin_{1,19df}$	ns	ns	7.2**	ns	ns	ns
$InjQuad_{1,19df}$	ns	ns	ns	ns	ns	ns
$Date_{1,19df}$	9.9***	ns	ns	8.7**	12**	13**
Date × $InjLin_{1,19df}$	23***	9.9**	ns	4.8*	4.4*	6.8*
Date × $InjQuad_{1,19df}$	12***	5.7*	ns	ns	ns	ns
(B) Base_Inj	10.5 ± 0.56**a**	0.081 ± 0.007**a**	153 ± 11**b**	0.46 ± 0.02**a**	0.63 ± 0.02**a**	183 ± 9.6**a**
1dPI_Inj	1.55 ± 0.29**b**	0.028 ± 0.005**b**	270 ± 17**a**	0.37 ± 0.01**c**	0.41 ± 0.02**c**	97 ± 7.0**c**
Base_Con	9.98 ± 0.62**a**	0.074 ± 0.005**a**	149 ± 8.2**b**	0.44 ± 0.02**ab**	0.62 ± 0.02**a**	176 ± 11**a**
1dPI_Con	8.73 ± 1.51**a**	0.063 ± 0.013**a**	171 ± 15**b**	0.40 ± 0.03**bc**	0.51 ± 0.04**b**	135 ± 18**b**

ns: $P > 0.05$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Several experiments have detected leaf P_n decreases after insect herbivory and mechanical injury on *Apocynaceae* spp. leaves [8,11,12,21,30; current study]. Constitutive and induced cardenolide investment has also been studied in many *Apocynaceae* spp. [25,43–45]. After leaf injury, P_n reductions have been measured in association with positive furanocoumarin induction [6], but in association with variable defensive cardenolide responses for *Asclepias* spp. [8]. Thus, additional studies with *N. oleander* and other *Apocynaceae* spp. may indicate whether positive cardenolide induction is associated with indirect photosynthesis reductions after herbivory [5–7,42]. It may also be useful to examine the extent to which specialist and generalist herbivores differentially influence leaf photosynthesis and chemical defense responses; again, several *Apocynaceae* spp. would be useful in such studies.

4.2. Injury: reduced light harvesting and photosynthetic non-stomatal limitation

All gas exchange and chlorophyll a fluorescence parameters (except C_i) in the two clipping experiments had significantly negative functions related to single leaf tissue loss intensity from clipping at 1 d PI. Increasing leaf injury intensity was also associated with quantitatively greater light harvesting reaction impairment as indicated by chlorophyll a fluorescence parameter responses [22]. Injury reduced F'_V/F'_M indicating that lower transfer efficiency of absorbed photon energy from reaction centers to PSII is irreversible (maximal light-adapted leaf photochemical efficiency), lower q_P meant that fewer oxidized PSII reaction centers at a given time were open to transfer absorbed photon energy to PSII, and ETR reduction indicated that light energy driving PSII per unit time appeared to be reduced [22]. The reduction in g_s but no net change in C_i in the 2004 clipping experiment suggests stomatal limitation to photosynthesis [46] followed by downregulation of light harvesting. However, reduction in g_s and a significant 1.5 h PI increase in C_i in the 2005 clipping experiment suggest photosynthetic non-stomatal limitation [46], where reduced PSII light harvesting may have led to stomatal closure. Increases in leaf C_i have also been measured in other studies after midrib cutting injury (very severe type of leaf injury) on *N. oleander* [21] and *A. syriaca* [11] leaves. Results from the 2004 clipping experiment showed incomplete 3 d PI recovery despite light harvesting and g_s recovery, while results from the 2005 clipping experiment suggest that P_n reduction at 3 d PI was associated with light harvesting reductions. Because dark-adapted leaf chlorophyll a fluorescence measurements were not also collected the influence of non-photochemical quenching (e.g., heat dissipation) on injured leaf photosynthesis could not be determined.

The 2005 SAW experiment provides an example of what happens to *N. oleander* leaf photosynthesis when herbivory occurs. Both P_n and g_s had negative exponential functions with injury intensity at 1 d post SAW herbivory indicating large indirect reductions. Surprisingly, injured leaf C_i had a significantly positive function with injury intensity at 1 d PI (only at 1.5 h PI in the 2005 clipping experiment), again suggesting non-stomatal limitation to photosynthesis [46] after SAW herbivory. The chlorophyll a fluorescence parameters had weakly significant negative linear functions with injury intensity at 1 d post-injury (F'_V/F'_M, q_P, and ETR). Yet, this did not mean that SAW feeding had no effect on these parameters. Herbivory had a qualitative effect on all photosynthetic parameters, where the average decrease (or increase for C_i) between pre-injury and 1 d PI measurements was significantly greater for leaves with SAW injury than control leaves (0% tissue loss). Some individual uninjured leaves had decreases (or increase for C_i for one leaf) at 1 d PI, likely in response to other environmental factors (e.g., light and temperature) between two measurement days. Yet, more leaves with SAW herbivory had larger changes with all parameters than uninjured leaves. An average C_i increase of 80%, and decreases of F'_V/F'_M, q_P, and ETR in leaves with SAW feeding provides additional support that PSII light harvesting impairment may have led to stomatal closure, so that indirect P_n reductions were due to non-stomatal photosynthetic limitation.

Insect herbivory has caused variable effects on leaf chlorophyll fluorescence parameters across plant species following injury. Significant F_V/F_M, F'_V/F'_M, and/or quantum efficiency (ΦPSII) reductions have been detected after insect herbivory [6,12,21,26,28,29], or only a small tissue ring around lost tissue [23]. In contrast, overcompensatory photosynthetic light reaction responses have been reported as increased F_V/F_M and/or ΦPSII after mechanical [47] or insect herbivory [48–50]. Thus, more needs to be understood about the factors that influence leaf indirect gas exchange and chlorophyll a fluorescence responses to defoliation and why impairment and overcompensatory responses occur in only some plant species after leaf tissue consumption by insect herbivores. Also, most studies do not explicitly consider how the amount of mechanical leaf injury or insect herbivory influences the degree of chlorophyll a fluorescence parameter changes after injury. The experiments reported in the current study suggest that injury intensity can be important for understanding what degree of indirect reductions are expected for a given level of leaf injury and thus the number of leaves needed to be measured to provide sufficient power to detect such differences.

4.3. Conclusions

Results with clipping injury and SAW herbivory on *N. oleander* leaves showed variation in leaf photosynthetic impairment to injury based on photosynthetic injury response curves. In two studies, leaf photosynthetic activity had disproportionately large decreases (or increase for C_i) per unit of tissue loss at low levels. Small amounts of *N. oleander* tissue loss on a single leaf could have a disproportionately large influence on the remaining leaf tissue in terms of photosynthetic activity. Thus, individual *N. oleander* leaves have low photosynthetic tolerance to injury, but whether this is due to high constitutive [but see 8] or induced chemical defense investment requires further study. In particular, examining leaf damage response curves for total cardenolide induction as well as induction of specific cardenolides like oleandrin and neriine would be useful, since there have been no studies on cardenolide induction after herbivory with *N. oleander*. Whether photosynthetic damage response curves correspond to possible cardenolide induction damage response curves could be useful for suggesting whether PI investment in chemical defense leads to a trade-off with maintaining photosynthesis. Leaf photosynthesis results suggest that impairment results from mechanical aspects of injury, which suggests that an injury signal travels through a leaf. Other gas exchange and chlorophyll a fluorescence results suggest non-stomatal limitations like downregulation of light harvesting reactions cause photosynthetic impairment, and most photosynthetic parameters had responses linearly proportional (but sometimes non-linearly) to injury intensity. Also, results from one clipping experiment and the SAW experiment suggest the possibility that post-injury light harvesting reductions may lead to subsequent stomatal closure. In summary, this is the first report of a negative exponential P_n (and g_s) impairment function with leaf injury intensity. Thus, a small amount of herbivory led to disproportionately large *N. oleander* indirect P_n impairment to indicate photosynthesis was highly sensitive to leaf injury.

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